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Award Number: DAMD17-99-1-9206

TITLE: Ribozyme Targeting the Novel Fusion Junction of EGFRvIII in Breast Cancer

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REPORT DATE: July 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2003	Final (1 Jul 1999 - 30 Jun 2003)

4. TITLE AND SUBTITLE
Ribozyme Targeting the Novel Fusion Junction of EGFRvIII in Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9206

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

EGFRvIII is a tumor specific, ligand-independent, constitutively activated variant of the epidermal growth factor receptor. We have detected high frequency of EGFRvIII expression in breast cancer. We also detected high frequency of co-expression of wt-EGFR with EGFRvIII mRNA in the same invasive breast carcinoma. Expression of EGFRvIII by ribozyme revealed down-regulation of EGFRvIII expression and reduced the tumorigenicity in athymic nude mice. These results suggest that EGFRvIII plays a significant role in breast cancer cell proliferation.

14. SUBJECT TERMS
Breast cancer, ribozyme, EGFRvIII, tumorigenesis

15. NUMBER OF PAGES
25

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

EGFRvIII is a tumor specific, ligand-independent, constitutively active variant of the epidermal growth factor receptor (1-7). Its expression has been detected in gliomas and various other human malignancies, including breast cancer (8-15). However, no detectable level of EGFRvIII has been observed in adult tissues, including normal breast tissues (12, 15).

This is a final report of grant DAMD17-99-1-9206. In the original proposal, we proposed three aims; 1) To determine the expression of EGFRvIII and correlation with clinicopathological prognostic factors in human breast cancer specimens; 2) To test the hypothesis that over-expression of EGFRvIII is capable of increasing the malignancy and metastatic potential of breast cancer cells; and 3) To test the hypothesis that down-regulation of EGFRvIII expression in breast cancer cells will reduce the tumorigenicity. We have completed all the proposed aims and we have published two manuscripts. In this final report, we will summarize and out line the research findings. Detailed descriptions please see the attached two manuscripts. In addition to these two manuscripts, we also examined the frequency of EGFRvIII protein expression immunohistochemically in over seven hundred primary breast cancer specimens with a specific EGFRvIII mAb on both tissue microarray and paraffin embedded specimens. The following, we described the findings for the immunohistochemical analysis study.

BODY

High frequency of EGFRVIII expression in human primary breast cancer specimens

To evaluate the importance of EGFRvIII expression in human breast cancer, we examined the frequency of EGFRvIII protein expression immunohistochemically in paraffin embedded specimens. We examined EGFRvIII expression immunohistochemically in specimens from three different institutes, which including 196 patients' paraffin embedded breast tissue specimens, as well as tissue array specimens obtained from 612 cases of primary breast cancer specimens. All specimens were graded by pathologists and classified into three groups: 1. Normal and benign lesions; 2. Ductal Carinoma In Situ (DCIS); 3. Infiltrating carcinoma (including moderately differentiated and poorly differentiated invasive carcinoma). High frequency of EGFRvIII expression was observed in human invasive breast cancer. Statistical analysis were performed with combined all the invasive breast samples from three different institutes to compare the distribution of EGFRvIII expression in this set of invasive breast carcinoma. Total of 721 cases, 25% (178/721) detected weak staining of EGFRvIII, majority of them exhibited moderate EGFRvIII 42% (305/721) and 5% (35/721) of these invasive breast cancer detected strong EGFRvIII expression. As shown in Table 1, the invasive tissues from each institution have similar distributions of expression levels for EGFRvIII. Table 1 clearly indicates that EGFRvIII expression is associated with disease progression. The statistical analysis indicates the expression of EGFRvIII associated with more malignant breast tumors remains significant, as shown in Table 1 ($p=0.0001$). Among samples with negative expression, the percentages of each tissue type decreases with increasing disease status. The samples with positive expression levels have percentages that increase as

disease status increases. It is noteworthy (Strikingly), 88% (9/11) of lymph node specimens expressing EGFRvIII. This indicates that EGFRvIII expression is strongly associated with breast cancer progression. Since, Manitoba is the only institution that provided samples from all tissue types. The numbers of samples for each expression level from the Manitoba tissues alone are displayed in Table 2. EGFRvIII is significantly associated with increasing invasiveness ($p < 0.0001$) with positive expression occurring in no samples (0%) of normal tissue, 18 DCIS samples (47%), and 74 (76%) of the invasive cancer samples. Also notice specifically that the percentages of weak positive and positive increase with disease progression.

Table 1 Numbers of Invasive Cancer Samples Expressing EGFRvIII by Institutions

	Invasive Tissue			
	ALL	**LCC**	**MAN**	**NIH**
	N (%)	**N (%)**	**N (%)**	**N (%)**
EGFRvIII	**721 (100)**	**50 (100)**	**97 (100)**	**574 (100)**
Negative	203 (28)	18 (36)	23 (24)	162 (28)
Weak Positive	178 (25)	8 (16)	25 (26)	145 (25)
Positive	305 (42)	17 (34)	41 (42)	247 (43)
Strong Positive	35 (5)	7 (14)	8 (8)	20 (3)
Not Readable	38	0	0	38

[1]Percentages did not include samples that were not readable.

1. Lombardi Cancer Center (LCC)
2. Manitoba (MAN)
3. National Institute of Health (NIH)

Table 2. Numbers of Samples Expressing EGFRvIII by Expression Strength and Tissue Type for Samples from Manitoba

EGFRvIII expression in Manitoba's sample

Marker	Tissue Type				
	All	Normal	DCIS	Invasive	P-Value
	N (%)	N (%)	N (%)	N (%)	
	146 (100)	11 (100)	38 (100)	97 (100)	
EGFRvIII					**<0.0001**
Negative	53 (36)	11 (100)	20 (53)	23 (24)	
Weak Positive	33 (23)	0 (0)	7 (18)	25 (26)	
Positive	49 (34)	0 (0)	8 (21)	41 (42)	
Strong Positive	11 (8)	0 (0)	3 (8)	8 (8)	

EGFRvIII expression inversely correlated with PR status in breast carcinoma

ER and PR status are known prognostic or predictive factor in invasive breast carcinoma; we were interested to evaluate the correlate of EGFRvIII expression with ER and PR. Because, we did not obtain the ER/PR data from the set of NIH tissue micro array samples, we evaluated total of 137 cases from LCC and Manitoba samples. Table 3 provides the statistical analysis of ER and PR status and their association with EGFRvIII status. As illustrated in Table 3, for ER, EGFRvIII expression did not correlate with ER status in breast tumor. The EGFRvIII+/ER+ and EGFRvIII-/ER- were not statistically different from each other. However, we found an inverse correlation between EGFRvIII expression and PR expression. ($p = 0.02$). PR negative was seen in 47% (65/137) of the

total data set. We found a 78% rate of PR negative among EGFRvIII+ cases verses 22% rate among EGFRvIII- cases (p= 0.02). As expression of EGFRvIII increases, fewer samples express PR.

Table 3. Numbers of Tissue Samples with ER and PR Status Available by Institution

	Total	ER +	ER -	PR +	PR -
	N (%)	N (%)	N (%)	N (%)	N (%)
All Institutions	137 (100)	77 (100)	60 (100)	72 (100)	65 (100)
LCC	50 (36)	37 (48)	13 (22)	36 (50)	14 (22)
MAN	87 (64)	40 (52)	47 (78)	36 (50)	51 (78)
NIH	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Taken all together, a remarkable aspect of this present study is that we found that EGFRvIII expression is associated with more aggressive invasive breast cancer and EGFRvIII expression may indicate disease progression. We detected EGFRvIII expression in 47% of DCIS, 72% of invasive breast carcinoma and 88% of metastatic lymph node specimens. No expression of EGFRvIII was detected in normal/benign breast tissues. Collectively, these results provide the strong evidence that EGFRvIII could play a pivotal role in human breast cancer progression.

KEY RESEARCH ACCOMPLISHMENTS

- Generated a functional active ribozyme targeting the novel junction of EGFRvIII.
- Demonstrate the therapeutic efficacy of an anti-EGFRvIII hammerhead ribozyme targeting the endogenous EGFRvIII expression in human breast cancer cells *in vitro* and *in vivo*.
- Expression of EGFRvIII in breast cancer MCF-7 cells reveals constitutively activated EGFRvIII and induces autophosphorylation of ErbB-2.
- Expression of EGFRvIII in breast cancer MCF-7 cells enhances proliferation and increases the spectrum and potency of ligand-mediated proliferation *in vitro* and induces tumorigenicity *in vivo*.
- By utilization of laser capture microdissection/RT-PCR state of art tehcnology, we were able to detect high frequency of co-expression of EGFRwt with EGFRvIII mRNA in primary invasive breast carcinoma specimens.
- EGFR wt expression displays a menbrane staining, whereas the EGFRvIII exhibits a predominantly cytoplamic staining and some nuclear staining as well.
- EGFRvIII expression is associated with more aggressive invasive breast cancer and EGFRvIII expression may indicate disease progression.
- Detailed descriptions please see attached two manuscripts.

REPORTABLE OUTCOMES

We have published two manuscripts and three abstracts resulted from the award. Copies of these manuscripts are included in the appendix of this final report. I have acknowledged U.S. Army Medical Research and Materiel Command, Breast Cancer Research Program in all two manuscripts and three abstracts for the support of my research.

Original Articles:

1. Hong Ge, Xiaoqi Gong and ***Careen K. Tang**. (2002) High Incidence of Co-expression of EGFRwt and EGFRvIII Transcripts in Human Invasive Breast Cancer Tissue By Laser Capture Microdissection/RT-PCR. *International Journal of Cancer*, 98:357-361.

2. Luo X.Y, Gong X.Q. and **Tang C.K. *** "Ribozyme Targeting the Novel Junction of EGFRvIII mRNA Inhibits Breast Cancer Cell Proliferation and Tumorigenesis". (2003) *International Journal of Cancer*, 104,716-721.

Abstracts:

1. X. Q. Gong, D. K. Moscatello, A. J. Wong, M. E. Lippman, and **C. K. Tang**. "EGFRvIII Enhances Tumorigenicity in Human Breast Cancer". *In Proceedings of American Association for Cancer Research*, 2000, Vol. 41: #2907, pp. 456.

2. H. Ge, X. Q. Gong, M. E. Lippman, and **C. K. Tang.** "Profiling of Differentially Expressed Genes Mediated By EGFRvIII in Human Breast Cancer." *In Proceedings of American Association for Cancer Research*, 2000, Vol. 41: #2911, pp. 457.

3. Xunyi Luo, Xiaoqi Gong, Marc E. Lippman and **Careen K. Tang.** "Ribozyme Targeting of Deletion-Mutant Epidermal Growth Factor Receptor Messenger RNA Inhibits Growth of Breast Cancer Cells *in vitro* and *in vivo*" *In Proceedings of American Association for Cancer Research*, 2001, Vol. 42:#3374, pp.628.

CONCLUSION

To our knowledge, the accomplished results from this proposal are original. These studies provided the first evidence that EGFRvIII play a pivotal role in breast cancer progression. It is the first report that high frequency of co-expression wild-type EGFR with EGFRvIII in primary invasive breast carcinomas (Manuscript published in IJC (2002)). Furthermore, it is also the first demonstration of the therapeutic efficacy of an anti-EGFRvIII hammerhead ribozyme targeting the endogenous EGFRvIII expression against human breast cancer cells *in vitro* and *in vivo* (Manuscript published in IJC (2003)). These results have provided valuable insights in the role of EGFRvIII in breast cancer. These studies have been led to a new direction of breast cancer research. Therefore, we have successfully accomplished the all the goals outlined in the proposed research project. We are grateful to the U.S. Army Medical Research and Material Command, Breast Cancer Research Program for the research support of this proposal.

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Int. J. Cancer: 98, 357–361 (2002)

DOI 10.1002/ijc.10224

Publication of the International Union Against Cancer

EVIDENCE OF HIGH INCIDENCE OF EGFRvIII EXPRESSION AND COEXPRESSION WITH EGFR IN HUMAN INVASIVE BREAST CANCER BY LASER CAPTURE MICRODISSECTION AND IMMUNOHISTOCHEMICAL ANALYSIS

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EGFRvIII was first reported in human glioblastomas. Subsequent reports indicated EGFRvIII protein to be frequently detected in several other human cancers, but not in normal tissues. Our previous studies suggested that EGFRvIII could induce a transformation from ligand-dependent non-tumorigenic cell line to ligand-independent malignant phenotype cells *in vitro* and *in vivo*. Transfection of EGFRvIII in MCF-7 cell line resulted in a 3-fold increase in colony formation and significantly enhanced tumorigenicity in nude mice ($p < 0.001$). EGFRvIII could also induce ErbB-2 phosphorylation. The existence and significance of EGFRvIII transcript in human breast cancer, however, was not reported. In our study, we detected the presence of EGFRvIII mRNA and revealed a high incidence (67.8%) of EGFRvIII transcript in human primary invasive breast cancer by utilizing laser capture microdissection (LCM)/RT-PCR to capture pure breast cancer cells. In addition, 57.1% of the infiltrating breast carcinomas expressed both EGFRwt and EGFRvIII mRNA in the same tumor. There is no detectable EGFRvIII mRNA in normal breast tissue. Evaluation of the EGFRwt and EGFRvIII protein levels in the same sample sets by immunohistochemical analysis further confirmed the LCM/RT-PCR finding. Our study provides first direct evidence of high incidence of coexpression of EGFRvIII and EGFRwt in human invasive breast cancer tissue. The unique characteristics and high prevalence of EGFRvIII in invasive human breast cancer as well as negative expression in normal breast may suggest its important role in breast carcinogenesis and make it an ideally potential target for treatment of breast cancer without interrupting normal EGFR signaling.

Key words: *EGFRvIII; EGFR; LCM; breast cancer*

Development and progression of breast cancer may result from an accumulation of varied aberrations of genes and their products. Detecting and understanding important molecules will address their roles in carcinogenesis and may be considered to be the prerequisite for the genesis of molecular therapeutics in breast cancer treatment. Overexpression of epidermal growth factor receptor (EGFR) has been detected in a variety of human cancers with 15–90% detection rates, and therefore, it has been thought a possible candidate for cancer therapy.[1–6] A relationship between overexpression of EGFR and increased metastatic potential and poor prognosis in breast cancers has been reported.[7,8] EGFRvIII, the most common deletion receptor of EGFRwt, was first reported in human glioblastomas.[9–11] Subsequently, a few reports demonstrated that EGFRvIII protein was also detected in other human cancers, including breast, ovarian, lung, and medulloblastomas,[12,13] but not in normal tissue.[13,14] In our previous studies, we have observed a high incidence of EGFRvIII protein (62%) in human invasive breast cancer and demonstrated a significantly enhanced tumorigenicity in EGFRvIII transfected MCF-7 breast cancer cells in nude mice.[15,16] Studies to confirm the presence and significance of EGFRvIII transcript and protein in breast cancer, however, are still underdeveloped.

To further confirm our early immunohistochemistry results and to ascertain EGFRvIII mRNA expression in human breast cancer tissue to explore the possible role of EGFRvIII in breast cancer genetics, we applied the laser capture microdissection (LCM) combined with RT-PCR detection. The PixCell LCM, a newly developed state-of-the-art technique, allows precise capture of specific cells from tissues comprising mixed cell types.[17,18] LCM can procure pure cell populations for molecular analysis by adhering the selected cells to a thermoplastic film through laser pulse.[17–20] By applying this technique, we were able to preferentially select "pure" breast cancer cells from mixed cell populations of breast tissues for genetic study. As a result, we detected a high incidence (67.8%) of EGFRvIII transcripts in primary invasive breast cancers by LCM but not in normal breast tissue. These results confirmed the presence of EGFRvIII in human breast cancer. We believe that these findings will have significant implications in breast cancer research, diagnosis, and treatment.

MATERIAL AND METHODS

Tissue collection and laser capture microdissection

Representative sections of breast cancer tissues from 28 patients were collected and frozen in cryo vials by submersion in liquid nitrogen and stored at −80°C. Frozen tissues were cut as 5 μm sections and mounted on plain glass slides. The slides were then hematoxylin-eosin (HE) stained and dehydrated in graded alcohol and xylene. Laser capture microdissection (LCM) was performed on the stained sections using a PixCell laser capture microscope (Arcturus Engineering Inc., Mountain View, CA) as described previously.[17–19] The tissue section was overlaid with a thermoplastic polymer membrane mounted on optically transparent caps. Cancer cells were captured by focal melting of the membrane through laser activation.

RNA extraction and RT-PCR analysis of EGFRvIII and EGFR

After microdissection, the cap with laser captured tiny tissue was put in a microtube. Total RNA was isolated by using a Micro RNA Isolation Kit (Stratagene, La Jolla, CA). The RNA was resuspended in 13.5 μl DEPC-H_2O. cDNA synthesis was performed at 42°C for 50 min with 12.5 μl (0.2–1 μg) of RNA, 2.5 μmol/L random hexamers, and 100 U of SuperScript II (Life Technologies, Inc., Bethesda, MD) in a final volume of 20 μl. PCR was performed with Taq DNA polymerase (Life Technologies, Inc.). Samples were incubated at 94°C for 3 min and followed by 30–35 cycles at 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min. A final extension was added at 72°C for 7 min. PCR products

Grant sponsor: United States Army; Grant number: DAMD 17-99-1-9206; Grant sponsor: Lombardi Cancer Research Center Histopathology, Macromolecular Synthesis/Sequencing and Microscopy/Imaging Shared Resource Facility; Grant number: P30-CA-51008.

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Received 6 August 2001; Revised 19 October 2001; Accepted 13 November 2001

Published online 15 January 2002

were revealed on agarose gel or re-amplified by a nested 3′ primer and the same 5′ primer. The sequences of the PCR primers are: 5′-ATGCGACCCTCCGGGACG-3′ (forward), 5′-GAGTATGT-GTGAAGGAGT-3′ (reverse) and 5′-GGGAAGCTTTCCGTTA-CACACTTTGCG-3′ (nested primer). Human GAPDH primers were used as an internal control.

Subcloning the RT-PCR product and sequence analysis

RT-PCR product was directly subcloned into pcDNA 3.1 vector by using TA Cloning kit (Invitrogen, Carlsbad, CA). The cloning reaction includes 1 μl of vector, 5 μl of RT-PCR product, 1 μl of T4 ligase and 10× ligase buffer and the total volume adjusted to 10 μl for 16°C, over night. The product was transformed into *E. coli* competent cells and mini-plasmid preparation was performed. Sequence analysis was done in automated sequencer with T7-primer.

Immunohistochemistry

Paraffin-embedded sections of primary invasive tumors were deparaffinized in xylene for 5 min. For frozen sections, acetone was used for fixation. After a brief rinse in 1× phosphate-buffered saline (PBS), the specimens were then subjected to staining. Two different specific antibodies were used for our study. One of them is specific for wildtype EGFR (Ab-3, Neomarker, CA) for frozen section only and the other one is specific for EGFRvIII (Ab-18, Neomarker) for paraffin section only. Their specificity were assessed by utilizing EGFR or EGFRvIII transfected NIH3T3 cells, as well as breast cancer cell lines. For paraffin embedded specimens, we used the anti-EGFRvIII (Ab-18) monoclonal antibody, which appears to recognize EGFRvIII only, but not wildtype EGFR. For frozen sections, we used an anti-EGFR (Ab-3) monoclonal antibody, which only reacts with wildtype EGFR, but not EGFRvIII. Both EGFR (Ab-3) and EGFRvIII (Ab-18) antibodies do not recognize other EGF-family receptors. After washing with PBS, a horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) secondary antibody (Kirkegard & Perry Lab. Gaithersburg, MD) was used at a dilution of 1/250 for 30 min. Finally, color was developed using diaminobenzidine (DAB) (BioGenex, San Ramon, CA) and section were then counterstained with hematoxylin (VWR).

RESULTS

Laser capture of breast cancer cells

In our study, a total of 28 cases of primary invasive breast cancers were subjected to LCM/RT-PCR analysis. Breast cancer cells were captured from breast cancer tissues by laser transfer from modified hematoxylin-eosin (HE) stained specimens. Figure 1 illustrates laser transfer of breast cancer cells from 2 representative cases. Case 1 (Fig. 1*a–c*) is a poorly differentiated breast carcinoma, and the cancer cells infiltrated into normal stroma network. LCM was able to successfully microdissect the individual cancer cells from tumor tissue. For Case 2 (Fig. 1*d–f*), a nest of infiltrated cancer cells surrounded by non-tumor tissue were transferred. Thus, LCM specifically captures only the cancer cells and avoids the surrounding stroma elements.

Identification of EGFRvIII and coexpression of EGFR/EGFRvIII mRNA in breast cancer tissues

The laser captured cancer cells were subsequently subjected to RNA isolation, followed by reverse transcription and nested PCR amplifications. A control experiment was performed to verify the specificity of the primers for PCR amplification (Fig. 2*a*). RNAs from our previous EGFR and EGFRvIII transfected hematopoietic cells (32D cells), as well as EGFRvIII transfected human breast cancer cell line MCF-7 cells were used as positive controls.[15] RNA from ErbB-2 and ErbB-3 cotransfected 32D cells was used as negative control. As expected, the wildtype EGFR mRNA displayed a 1026-bp fragment and the EGFRvIII mRNA displayed a shortened 225-bp fragment. No PCR products were detected from ErbB-2+ErbB-3 transfected 32D cells. A normal breast tissue from a 42-year-old woman was used to determine whether or not EGFRwt or EGFRvIII were expressed. Figure 2*b* depicts the normal breast tissue to express neither wildtype nor mutant form of EGFR.

TABLE I – SUMMARY OF DETECTION OF EGFR mRNA AND EGFRvIII mRNA EXPRESSION IN HUMAN PRIMARY INFILTRATING BREAST CARCINOMA BY LCM/RT-PCR TECHNOLOGY

Expression of EGFRmRNA or EGFRvIII mRNA	No. of Positive/ Total of Evaluated
None	1/28 (3.6%)
EGFR wild-type	8/28 (28.6%)
EGFRvIII	3/28 (10.7%)
EGFR wild-type + EGFRvIII	16/28 (57.1%)

In these 28 cases of invasive breast cancer specimens, we observed some to express EGFR wt mRNA only, and others to express the mutant form EGFRvIII. Interestingly, a majority of breast cancer tissues detected both mutant and wildtype EGFR mRNA in the same tumor. Figure 2*c* demonstrated representative results of the invasive breast carcinoma tissue samples for EGFRwt mRNA only (Case 200), mutant form EGFRvIII (Case 531) and coexpression of EGFR/EGFRvIII (Cases 657 and 680). These results indicate the presence of a heterozygote of this receptor in breast cancer. The heterozygotes displayed varied intensities in both wildtype and mutant forms. Table I summarizes the LCM/RT-PCR results. Wildtype EGFR mRNA was only detected in 28.6% (8/28) specimens of primary invasive breast cancers, whereas 10.7% (3/28) specimens expressed EGFRvIII mRNA only. The most revealing finding from this set of experiments was that, 57.1% (16/28) of the infiltrating breast carcinomas expressed both EGFRwt and EGFRvIII mRNA in the same breast tumor. Only 1 case expressed neither detectable levels of EGFRwt nor EGFRvIII by RT-PCR. The GAPDH in this case, however, was detected with similar levels as in all other cases.

Verification of the EGFRvIII expression in breast cancer

To further identify and verify the deletion junction sequence of the LCM/RT-PCR product, the 225-bp EGFRvIII fragment was subcloned and sequenced. The sequence of this 225-bp fragment derived from infiltrating breast carcinoma matched the published EGFRvIII sequence with a 801 bp deletion in the extracellular ligand binding domain, creating a glycine at the splicing position as that noted in human glioblastoma (Fig. 2*d,e*).

Detection of EGFRvIII and EGFR protein coexpression in breast cancer

The same set of primary invasive breast cancer specimens, subjected to LCM/RT-PCR studies, were used to assess the protein levels of EGFRwt and EGFRvIII by immunohistochemical (IHC) analysis. We have characterized a specific EGFRvIII (Ab-18) monoclonal antibody. As shown in Table II, antibody EGFRvIII (Ab-18) specific recognizes EGFRvIII but not the wildtype EGFR protein. In addition, this antibody does not cross react with other EGF-family receptors (Table II). Therefore, this specific antibody was used for all the immunohistochemical analysis studies in our study. We also characterized a panel of commercially available EGFR antibodies. EGFR (Ab-3) monoclonal antibody appears only recognizes the wildtype EGFR but not the EGFRvIII receptor. This EGFR (Ab-3) does not cross react with other EGF-family receptors (Table II).

A total of 21 cases of frozen sections of breast cancer tissues were available for determination of EGFRwt expression with this well-characterized specific anti-EGFRwt monoclonal antibody (Ab-3). As summarized in Table III, EGFR wt expression was detected in 61.9% (13/21) specimens of primary invasive breast cancers, most of which displayed membrane immunoreactivity of variable intensities (Fig. 3*a*). There was no cytoplasmic reactivity noted in EGFRwt staining. The same set of mirror image paraffin embedded tissues was also used to analyze EGFRvIII expression

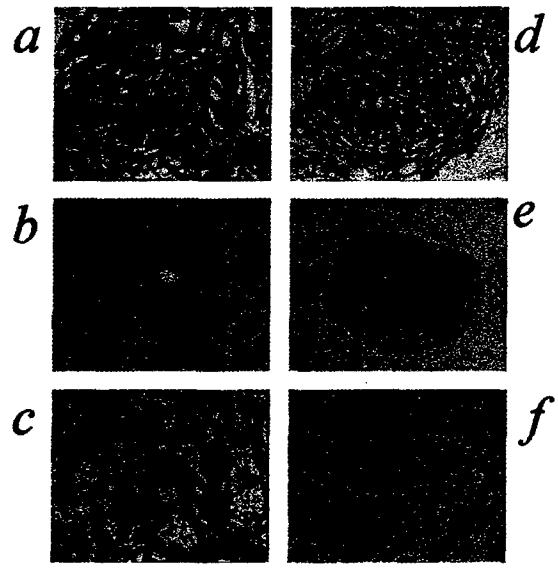

FIGURE 1 – LCM transfer of human breast cancer cells from frozen tissues. (*a,d*) Two images of infiltrated breast cancer sections. (*b,e*) Images of transferred cancer cells onto polymer films of Capture. (*c,f*) Surrounding tissues that remain on slides after LCM.

with EGFRvIII monoclonal antibody (Ab-18). EGFRvIII expression was detected in 57.8% (11/19) specimens (Table III). EGFRvIII, however, displayed an intracellular localization in contrast to the plasma membrane location of the wildtype EGFR (Fig. 3*b*). Overall, the immunostaining results were in concordance with LCM/RT-PCR data (Table IV). Comparing the wildtype EGFR mRNA to EGFR protein, 14 of 21 cases were exactly matched (Table IV). Six out of 21 wildtype cases were detected only at the mRNA level, but no increased protein content by immunological evaluation. A similar observation resulted from the comparison of EGFRvIII mRNA to EGFRvIII protein (Table IV). It seems that LCM/RT-PCR is more sensitive than immunostaining as expected.

DISCUSSION

In our study, by applying LCM technique, which allows us to exclusively obtain the pure breast cancer cells from mixed cell populations of breast tissues, we definitively demonstrate the existence of EGFRvIII mRNA in human invasive breast cancer tissue. EGFRvIII is a ligand-independent, constitutively active variant of the epidermal growth factor receptor. Its expression has been detected in many human malignancies including breast cancer. Our previous studies and reports by others have shown that EGFRvIII protein is detected in over 60% of primary invasive breast cancer.[15,20] Our early studies also clearly demonstrated EGFRvIII to be capable of transforming a non-tumorigenic, IL-3-dependent murine hematopoietic cell line (32D cells) into an IL-3-independent and ligand-independent malignant phenotype *in vitro* and *in vivo*. Such profound transforming activity has not been observed with any homo- or hetero-dimers of wildtype ErbB-family receptors in this system.[16] Transfection and expression of EGFRvIII in human breast cancer cell line (MCF-7) induced approximately a 3-fold increase in colony formation and significantly enhanced tumorigenicity of MCF-7 cells in athymic nude mice ($p < 0.001$).[16] In addition, ErbB-2 phosphorylation was enhanced in EGFRvIII transfected MCF-7 cells, which indicated that EGFRvIII could activate ErbB-2 kinase activity.[16] Collectively, these results provide the direct evidence that EGFRvIII could play a pivotal role in human breast cancer genetics. To confirm the presence of EGFRvIII in breast cancer clinical specimens, we utilized the Laser Capture Microdissection/RT-PCR, to successfully and clearly demonstrate the existence of EGFRvIII

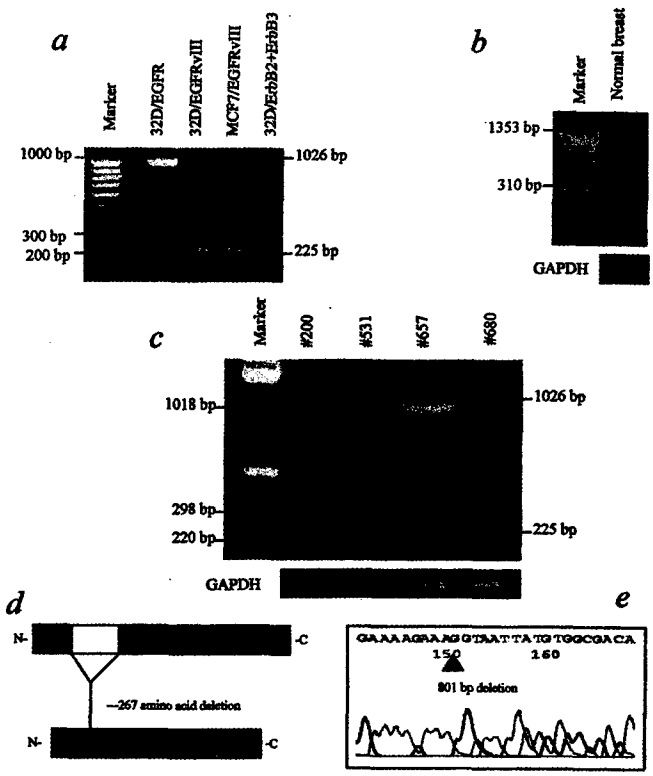

FIGURE 2 – (*a*) A control experiment for EGFRvIII and EGFR in cell lines. RNAs isolated from EGFRvIII and EGFR transfected 32D cells, as well as EGFRvIII transfected MCF-7 cells were used as positive controls. RNAs isolated from ErbB-2 and ErbB-3 cotransfected 32D cells were used as a negative control. RT-PCR and nest PCR were performed for these samples. As illustrated in this ethidium bromide gel, the EGFR mRNA (1026 bp) and EGFRvIII mRNA (225 bp) was detected in EGFR and EGFRvIII transfected 32D cell lines, respectively. No products were detected in ErbB-2+ ErbB-3 transfected 32D cells. (*b*) Normal breast tissue do not express detectable levels of EGFR and EGFRvIII. (*c*) Detection of EGFRvIII mRNA in primary infiltrating breast cancer specimens. RNA were extracted from Laser Capture Microdissected breast cancer tissues and subjected to RT-PCR and nest PCR. Case 200 exhibited a 1026 bp product (wildtype EGFR). Case 531 contained a 225 bp product (EGFRvIII), whereas Case 657 and 680 contained both 1026 bp (wildtype EGFR) and 225 bp (EGFRvIII) products. (*d*) Schematic representation of the structure of EGFR and EGFRvIII gene. EGFRvIII shows a 801 bp (267 amino acid) deletion in the extracellular domain of the EGFR gene. (*e*) Sequence analysis of a 225-bp RT-PCR fragment from LCM of human breast cancer specimen confirms the deletion sequence of EGFRvIII. The triangle indicates the deletion junction sequence.

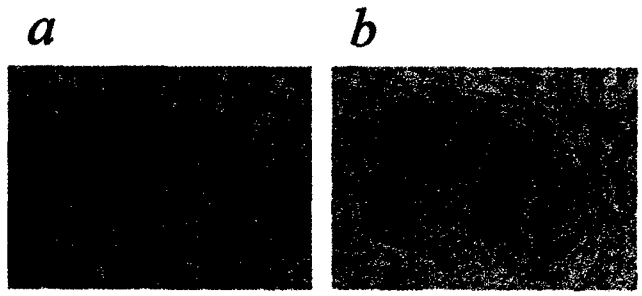

FIGURE 3 – A representative of immunochemical analysis of primary human infiltrated breast carcinoma specimens with specific antibodies against EGFR or EGFRvIII. Counterstaining with hematoxylin were used for viewing negative stained cells (blue). (*a*) EGFR immunoreactivity shows in cellular membranes (brown). (*b*) EGFRvIII positive stained cells shows in cytoplasms.

TABLE II – SPECIFICITY OF THE EGFR AND EGFRvIII ANTIBODIES

Cell line	EGFR	ErbB-2	ErbB-3	ErbB-4	EGFRvIII	Reactivity with αEGFRvIII(Ab-18)	Reactivity with αEGFR (Ab-3)
NIH/EGFR	++++	–	–	–	–	–	+
NIH/EGFRvIII	–	–	–	–	++++	+	–
MCF-7	–/+	++	+++	++++	–	–	–
MCF7/EGFRvIII	–/+	++	+++	++++	++++	+	–
MDA-MB-468	++++	++	+	–	–	–	+
MDA-MB-453	–	++++	+++	+	–	–	–

TABLE III – SUMMARY OF EGFR AND EGFRvIII mRNA AND PROTEINS EXPRESSIONS IN HUMAN BREAST CANCER SPECIMENS BY LCM/RT-PCR AND IMMUNOCHEMICAL ANALYSIS

Expression (mRNA or proteins)	LCM/RT-PCR % (# positive/ total # of evaluated)	IHC %(# positive/ total # of evaluated)
EGFR wt	85.7% (24/28)	61.9% (13/21)
EGFRvIII	67.8% (19/28)	57.8% (11/19)

mRNA in breast cancer tissues. Due to the tiny tissue obtained from LCM, we found in some cases, that wildtype or mutant fragments were difficult to detect in the first PCR amplification. The second nest PCR, however, was helpful in revealing both wildtype and mutant bands. The design of a nest PCR contributed greatly to demonstrate coexpression of wildtype and mutant receptors of EGFR. No detectable levels of EGFRvIII could be detected in 1 normal breast tissue even with a nested PCR. The high prevalence of EGFRvIII in invasive human breast cancer supports that EGFRvIII may play an important role in breast cancer progression. Moreover, the coexistence of EGFRvIII and FGER mRNA in the same tumor at such a high percentage [57.1% (16/28)] of the infiltrating breast carcinomas in breast cancer cells was unexpected (Fig. 2*c*, Cases 657 and 680). In addition, the intensities of the RT-PCR bands between the EGFRwt and the EGFRvIII fragments were varied in different cases, it will be interesting to quantitatively verify whether the alteration of this receptor is associated with progress of the disease.

We further assessed the coexpression of EGFR and EGFRvIII at the protein level by immunohistochemical (IHC) analysis with specific antibodies against EGFR or EGFRvIII in the same set of breast cancer specimens. Overall, the results correlated well with LCM/RT-PCR. Several cases show definitely positive results by LCM/RT-PCR, but relatively faint or no obvious staining by IHC analysis. It is reasonable that LCM/RT-PCR is more sensitive than immunostaining (Table III), because polymerase chain reaction is a very efficient method in amplifying gene, whereas immunohistochemistry detection only reveals the protein, which is overexpressed. There are 2 cases showing discrepant results (Cases 283 and 312). We are not sure of the reason for these discrepancies. Reports from early studies regard the EGFR overexpression in human breast cancer varies between 15–90%. As we now know that most commercially available antibodies to EGFR are able to recognize both wildtype and mutant EGFR that raises a concern on the antibody selection. Therefore, some previous publications on the interpretations of EGFR overexpression in breast cancer may be questionable, in which the overexpression may only attribute to the wildtype EGFR, and the detection of EGFRvIII was overlooked. In brain tumor, it was found that EGFR overexpression was associated with a mutant form of EGFR,[10,11] with the most common mutant form of EGFRvIII.[21] These results support our present findings. Although the double staining is not available in our study due to the limitation in the application of antibodies, our LCM/RT-PCR result is a strong support for co-presence of EGFRvIII and EGFR. Hence, our present studies provide information that spontaneous alteration of EGFR mRNA occurs in the breast cancer. The coexistence of EGFR and EGFRvIII in cancer cells may reflect transitions or progressions of tumor behavior. To our knowledge, this is the first observation for EGFRvIII/EGFRwt coexpressing in breast cancer.

TABLE IV – COMPARISON OF EGFR AND EGFRvIII mRNA AND PROTEIN EXPRESSION BY LCM/RT-PCR AND ROUTINE IHC TECHNIQUES IN HUMAN BREAST CANCER SPECIMENS

Case	EGFR mRNA (LCM/RT-PCR)	EGFR protein (IHC)	EGFRvIII mRNA (LCM/RT-PCR)	EGFRvIII protein (IHC)
181	+	ND[1]	–	–
191	–	ND	+	–/+
194	–	ND	+	–/+
200	+	+/–	–	–
212	+	–[2]	–	–
218	+	+	–	ND
283	+	+	–	+
312	–	+	–	+
323	+	+	+	+
330	+	+	–	–
368	+	+/–	+	–
403	+	–[2]	+	+
474	+	+/–	+	+
493	+	–[2]	+	+
508	+	+	+	+
515	+	+/–	+	ND
523	+	–[2]	+	ND
525	+	ND	+	+
531	±~+	–~±	+	+
543	+	±[2]	+	+
617	+	–	+	ND
657	+	+	+	ND
680	+	+	+	ND
883	–	–	+	+
897	+	+	–	–

[1]ND, not valuable.—[2]Only a few cells (<1%) show positive staining.

Despite the high frequency of EGFRvIII expression in primary invasive breast cancer specimens, we observed that most of these cell lines express EGFRvIII *in vivo*, but not it *in vitro*. *In vitro*, the cells were cultured in IMEM, supplemented with 10% FBS and 1% glutamine at 37°C, 5% CO_2 incubator as published elsewhere. Obviously, it is not due to technical problem in culture. Similar findings were also reported in human glioblastoma. Virtually, all cell lines derived from primary glioma tumor lose EGFRvIII expression in tissue culture.[22] It was suggested that either a growth disadvantage *in vitro* or a selection for EGFRvIII overexpression *in vivo*.[23] It is very possible that there are some critical factors, which might facilitate the EGFRvIII expression *in vivo*, but that are missing *in vitro*.

It has been well documented that the EGFRwt is a membrane receptor,[24] whereas EGFRvIII mainly expresses in the cytoplasmic in breast tumor tissues. Similar observations have been reported in human glioblastoma tumors, where EGFRvIII is expressed exclusively in the cytoplasm.[25] As shown in Figure 3, the EGFRwt displays a membrane staining, whereas the EGFRvIII exhibits a predominantly cytoplasmic staining and some nuclear staining as well in primary invasive breast cancer tissues. The altered subcellular location of EGFRvIII in breast cancer may suggest that the trafficking, signaling, recycling, as well as degradation of the mutant receptor may somehow differ from its wildtype form and leads to cytoplasmic location. The changed subcellular location may attribute to a sustained tyrosine kinase activity, which manifests a more potent, aggressive and oncogenic effect in human cancer. Recent report also indicated that EGFRvIII receptor-recep-

tor self-association is highly dependent on a conformation induced by N-linked glycosylation. Ligand-independent dimerization of the EGFRvIII is contingent upon core glycosylation.[26] Phosphorylation-induced conformation change results in exposure of sequence motifs involved in endocytic and lysosomal sorting and such unmasking is thought to be obligatory for receptor downregulation.[27] Further functional and biological analysis, as well as trafficking and signaling is likely to provide comprehensive insights for the role of EGFRvIII in breast cancer.

LCM is a powerful technology. It exclusively obtained the pure breast cancer cells from mixed cell populations of breast tissues. Other techniques, such as Southern or Northern Blotting and RNA protection assay will only provide the information from whole breast tissue, but not from pure cancer cells, which may make it difficult to interpret result. This issue is avoided by LCM selection for exclusive malignant cell, and the result is more accurate. In addition, because RNA is likely to be degraded through prolonged exposure at room temperature after a series of experimental procedures including tissue cutting, staining, and microdissection, most LCM/RT-PCR applications reported the amplification of fragments around 500 bp.[19,20,27] In contrast, we successfully amplified a 1026-bp fragment, whose fragment size is relatively difficult to be detected by LCM/RT-PCR technology due to impaired starting materials.

In summary, our study provides the evidence that a spontaneous alteration of EGFR occurs in breast cancer. Coexpression of EGFRwt and EGFRvIII mRNA and protein are frequently detected in human primary invasive breast cancer. No detectable levels of EGFRvIII were detected in a normal breast tissue, which was tested. Although our samples are limited, the high percentage of EGFRvIII expression may implicate its possible role in the pathogenesis of breast cancer. In addition, we successfully amplified >1 kb fragments by LCM/RT-PCR, thus extending the fragment limit size reported by utilizing LCM/RT-PCR technology. Considering the high frequency of this receptor alteration, as well as the unique distributions of the receptor makes it a compelling target for therapeutic strategies without interfering with normal signaling. We believe that our study provides useful information on molecular genetics and potential target for breast cancer.

ACKNOWLEDGEMENTS

This work was supported by the United States Army Medical Research and Material Command Breast Cancer Grant DAMD 17-99-1-9206 (C.K.T.). We thank Dr. M.E. Lippman for helpful discussions and we express our appreciation to Dr. A. Hayes for technical suggestion in laser capture microdissection. Figures 1–3 were supported in part by the Lombardi Cancer Research Center Histopathology, Macromolecular Synthesis/Sequencing and Microscopy/Imaging Shared Resource Facility, U.S. Public Health Service Grant P30-CA-51008.

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Int. J. Cancer: **104,** 716–721 (2003)

Publication of the International Union Against Cancer

SUPPRESSION OF EGFRvIII-MEDIATED PROLIFERATION AND TUMORIGENESIS OF BREAST CANCER CELLS BY RIBOZYME

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EGFRvIII is a tumor specific, ligand-independent, constitutively active variant of the epidermal growth factor receptor. Its expression has been detected in many human malignancies including breast cancer. No detectable level of EGFRvIII has, however, been observed in adult tissues, including normal breast tissues. These unique features of the EGFRvIII make it an excellent target for biologically based therapies. We have designed and generated a tumor specific ribozyme targeted to EGFRvIII. This specific EGFRvIII ribozyme is able to effectively cleave EGFRvIII mRNA under physiological conditions in a cell-free system, but does not cleave wild-type EGFR and other EGF-family receptors. While expressing this EGFRvIII-ribozyme in breast cancer cells, EGFRvIII-ribozyme is capable of downregulating endogenous EGFRvIII expression at the mRNA and protein levels. Inhibition of proliferation was observed in EGFRvIII-ribozyme transfectants. In addition, downregulation of EGFRvIII in breast cancer cells significantly inhibited tumor growth in athymic nude mice. Furthermore, this ribozyme has no effect on EGF-family receptor expression or the proliferation of breast cancer cells, which do not express EGFRvIII but express wild-type EGFR and other EGF-family receptors. These results suggest that we have generated a tumor-specific, biologically functional ribozyme and further demonstrate that EGFRvIII plays a significant role in breast cancer cell proliferation. The ultimate goal of this approach is to provide a potential treatment for breast cancer by specifically targeting this receptor. © *2003 Wiley-Liss, Inc.*

Key words: *breast tumor; EGFRvIII; ribozyme; tumorigenesis*

Reports have demonstrated that spontaneous rearrangements within the EGFR molecule (EGFRvIII) arise in primary human brain tumors.[1,2] This EGFRvIII molecule also frequently exists in other human cancers, but has never been detected in normal tissue.[3–9] Our early studies showed that this novel EGFRvIII molecule is present in over 50% of primary breast carcinomas.[10] High incidence of co-expression of EGFRvIII and EGFRwt were detected in human invasive breast cancer tissue by Laser Microdissection/RT-PCR technology.[11] Neither EGFRvIII protein nor EGFRvIII mRNA was detected in normal breast tissue.[10,11] In addition, expression of EGFRvIII in human breast cancer cell line MCF-7 cells shows induction of proliferation and enhanced tumorigenicity in nude mice.[12] These unique characteristics of the EGFRvIII molecule make it an attractive candidate as a therapeutic target.

Ribozymes are catalytically active subsets of small RNA molecules that possess the property of site-specific cleavage of RNA substrates,[13] thereby intercepting gene expression by forestalling subsequent translation. The discovery of catalytic RNA molecules has led to the notion of using these ribozymes as therapeutic agents. The ability of a ribozyme to recognize and cleave a specific RNA target has attracted considerable interest, because it can be exploited to combat disease at the level of genetic information. We designed and generated a tumor specific hammerhead ribozyme targeted to the novel fusion junction of EGFRvIII. We demonstrated that this specific EGFRvIII ribozyme is able to effectively cleave EGFRvIII mRNA under physiological conditions in a cell-free system, but does not cleave wild-type EGFR and other EGF-family receptors. Furthermore, this EGFRvIII-ribozyme is capable of downregulating endogenous EGFRvIII expression at both mRNA and protein levels in MDA435/LCC6 breast cancer cells. Downregulation of EGFRvIII in MDA435/LCC6 breast cancer cells results in inhibition of proliferation, and reduction of tumorigenicity in athymic nude mice. Furthermore, this ribozyme has no effects on the expression of EGF-family receptors and proliferation in MCF-7/LCC2 breast cancer cells, which do not express EGFRvIII but express wild-type EGFR and other EGF-family receptors. These results suggest that we have generated a tumor-specific biologically functional ribozyme and further demonstrate that EGFRvIII plays a significant role in breast cancer cell proliferation.

MATERIAL AND METHODS

Cell lines and cell culture

MDA435/LCC-6, MCF-7/LCC-2[16,17] were generously provided by R. Clarke (Georgetown University Medical Center). MCF-7/EGFRvIII breast carcinoma cell lines[12] and their derivatives were maintained in IMEM (Cellgro), supplemented with 10% FBS (Biofluids, Rockville, MD).

Generation of EGFRvIII ribozyme

We selected the ribozyme target site the novel junction sequence of EGFRvIII mRNA, 5′-AAGAAAGGUAAUUAUGU-3′, where underlined nucleotides comprise the novel cleavage site for this ribozyme.

Plasmid construction

Two synthetic single-stranded ribozyme oligonucleotides were subcloned into the mammalian vector pCR3. The sequence and orientation of the inserts were confirmed by dideoxynucleotide sequencing of the construct using the Sequenase kit, version 2.0 (US Biochemical, Cleveland, OH). The EGFRvIII ribozyme sequence is: 5′-ACAUAAUCUGAUGAGUCCGUGAGGACG-AAACUUUCUU-3′. This ribozyme was then subcloned into pCDNA3.1/zeo vector and the sequence and orientation of the inserts were confirmed.

Ribozyme-mediated mRNA cleavage in vitro

The substrate EGFRvIII cDNA fragment was derived by PCR with EGFRvIII full cDNA, which was generously provided by A. Wong. The PCR primers for subcloning of this EGFRvIII fragment are: 5′ primer sequence CCTCCGTCTGAATTTTGCTTT and 3′ primer sequences GCCGCGTAGATTTCTAGGTT.

Grant sponsor: United States Army Medical Research and Material Command; Grant number: DAMD 17-99-1-9206; Grant sponsor: Susan G. Komen Breast Cancer Foundation 9806; Grant sponsor: Lombardi Cancer Research Center Flow Cytometry Core Facility; Grant sponsor: DHHS/NIH; Grant number: P30-CA-51008.

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Received 10 July 2002; Revised 18 September 2002; Accepted 10 December 2002

DOI 10.1002/ijc.11007
Published online 11 February 2003 in Wiley InterScience (www.interscience.wiley.com).

We then carried out *in vitro* run-off transcripts from an EGFRvIII cDNA construct to generate the EGFRvIII ribozyme substrate. Likewise, EGFRvIII-ribozyme was chemically synthesized as DNA oligonucleotide and subsequently synthesized *in vitro* by using the T7 RNA polymerase. Cleavage reactions were carried out in 50 mM Tris-HCL (pH 8.0) and 20 mM $MgCl_2$, Substrate and ribozyme transcripts were then mixed and incubated at 50°C for 30 min. Reaction products were analyzed on 6% urea polyacrylamide gel and products were detected by autoradiography.

Transfection

Cells (1×10^6) and 10–15 μg of plasmid DNA were used for each transfection. Transfection was carried out using the Calcium Phosphate Transfection System (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The cells were then selected in a growth medium containing appropriate amounts (350 μg/ml) of Geneticin (G418-sulfate; Invitrogen).

Autophosphorylation of EGFRvIII

The cells were serum starved overnight at 37°C prior cell lysis. Cells were lysed in HEPES lysis buffer (50 nM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, and 1 mM EGTA), and the cell debris was pelleted by centrifugation.[14]

The lysates were then subjected for immunoprecipitation with anti-EGFRvIII (NeoMarker, Union City, CA), in combination with Protein A-Sepharose CL-4B (Amersham Pharmacia, Sweden) overnight at 4°C with gentle agitation. Immunoprecipitates were then separated by SDS-PAGE and transferred to nitrocellulose. Bound proteins were immunoblotted with anti-phosphotyrosine monoclonal antibody (Upstate, Lake Placid, NY), followed by blots with 0.5 μg/ml of secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (ECL; Amersham).

Fluorescence-activated cell sorter (FACStar) analysis

Cells (1×10^6) were harvested and then stained for 1 hr with anti-EGFR (Ab-1) monoclonal antibody (NeoMarker) or anti-EGFRvIII monoclonal antibody (Ab-18) (NeoMarker), or ErbB-2, ErbB-3 (c-17) and ErbB-4 (c-18) (Santa Cruz Biotechnologies, Santa Cruz, CA) at 4°C. Stained cells were then washed with cold PBS. A secondary FITC-anti-mouse antibody was used, and the expression levels of EGFRvIII, as well as other EGF-family receptors in each cell lines were quantitatively measured by flow cytometry.

Semiquantitative PCR to assess EGFRvIII mRNA expression

The primers used for PCR were 5′-ATGCGACCCTCCGGGACG(18mer), 35′-GAGTATGTGTGAAGGACT(18mer). PCR was carried out in 50 μl volume with 1U of Taq DNA polymerase at 94°C for 45 sec, 52°C for 60 sec and 72°C for 90 sec. A series of PCR samples were then collected at different cycles, followed by a final extension step at 72°C for 15 min.

Total cellular RNAs from ribozyme transfectants were isolated using TRIZOL Reagent (Invitrogen). Equal concentrations of RNA were then subjected to RT with random primers. Total RNA derived from MDA435/LCC6/wt and MCF-7/LCC2/wt cells were used as controls. The resulting cDNAs were amplified on semiquantitative PCR by the primers for the respective construct and GAPDH as an internal control (reference gene).

Northern blot analysis

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen) followed by isopropanol precipitation. The RNA concentration was determined by measuring absorbance at 260 nm. Ten micrograms of total RNA was electrophoresed on formaldehyde containing 1% agarose gel and transferred the RNA onto a nylon membrane. ^{32}P-labeled EGFRvIII DNA probe was used for hybridization. 18S rRNA was used as an internal control (loading control).

Anchorage-dependent growth assays

Cells were harvested using trypsin, and 3,000 cells/well were plated in 24-well plates (Costar, Cambridge, MA). All samples were prepared in triplicate. Cells were counted in a Coulter Counter (Beckman Coulter, Inc., Palo Alto, CA) on Day 1 (the following day), and Days 3 and 7. Values indicate the mean of triplicate determinations.

Anchorage-independent growth assays

A bottom layer of 1 ml of IMEM containing 0.6% agar and 10% FBS was prepared in 35-mm tissue culture dishes. After the bottom layer solidified, cells (10,000/dish) were then added in a 0.8 ml top layer containing 0.4% Bacto Agar and 5% FBS. All samples were prepared in triplicate. Cells were incubated for 10 days at 37°C. Colonies larger than 60 μm were counted on a cell colony counter (Ommias 3600; Imagine Products Inc., Carmel, Indiana).

In *vivo studies*

Four- to 6-week-old female athymic nude mice were inoculated into the mammary fat pat with MDA435/LCC6/wt, MDA435/LCC6/vector, as well as EGFRvIII ribozyme-transfected clones, LCC6/RzC1 and LCC6/RzC9. Tumor size was measured twice weekly and calculated by measuring tumor volume (length × width × thickness). When tumor volumes reached up to 270 ± SD mm^3, mice were sacrificed. Xenograft tumors were surgically removed and snap frozen for Western blotting analysis of EGFRvIII expression.

Statistical analysis

A general estimating equation (GEE) procedure[22] was implemented to determine if the growth rate were different among the groups for the anchorage-dependent assay. The cell counts data were transformed by natural logs to conform to normality assumptions. For the anchorage-independent assay, the differences in colony numbers were tested using a 2-way analysis of variance,[23] with colony size and group as the factors. In addition, a Kruskal-Wallis test[24] was carried out to determine whether the groups differed in the proportion of colonies growing into different size group. For *in vivo* studies, a nested analysis of variance test was carried out to assessing the differences in tumor size.

RESULTS

Generation and demonstration of EGFRvIII ribozyme efficacy and specificity in a cell-free system

The unique characteristics of EGFRvIII receptor make it an attractive candidate as therapeutic target. Specifically, the deletion junction of EGFRvIII creates a new amino acid glycine via a unique GUA sequence that serves as a novel natural targeting site for hammerhead ribozyme. We designed a hammerhead ribozyme targeted to this novel fusion junction site within the EGFRvIII mRNA (Fig. 1). This EGFRvIII ribozyme should only cleave EGFRvIII mRNA but not the wild-type EGFR. The catalytic activity of experimental and control ribozymes was first evaluated in an extracellular system. As illustrated in Figure 2, this EGFRvIII ribozyme can cleave EGFRvIII mRNA precisely and efficiently under physiological conditions in a cell free system. Cleavage was specific as the actual sizes of the cleaved fragments corresponded to the expected sizes, when cleavage occurred immediately 3′ to the GUA sequence. As an efficacy control, catalytically inactive mutant ribozyme was engineered. The point mutation of G–A in the catalytic domain of this EGFRvIII ribozyme results in a loss of catalytic activity, as predicted by the mutational studies of McCall *et al.*[15] (data not shown). We also tested the specificity of this EGFRvIII ribozyme by using the wild-type EGFR mRNA as a substrate. As expected, no cleavage was observed by this ribozyme (data not shown). These results indicate that the GUA sequence that chosen in the novel fusion junction of EGFRvIII mRNA is accessible to ribozyme mediated cleavage in an extracellular system.

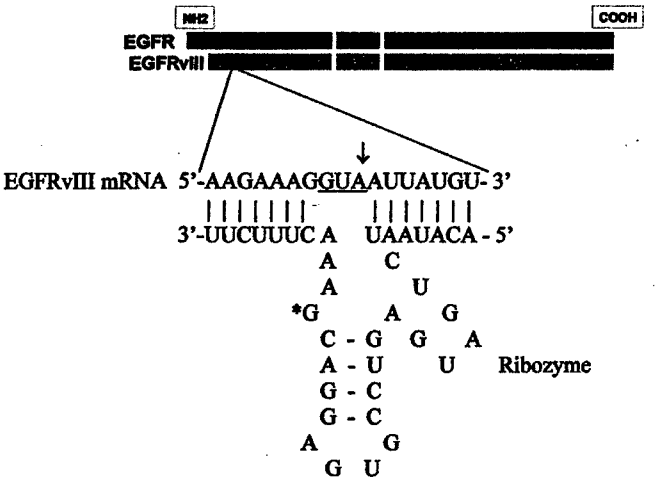

FIGURE 1 – Illustration of targeted sequences and predicted structure of the synthetic hammerhead ribozyme. This hammerhead ribozyme is targeting the novel deletion junction of EGFRvIII. The cleavage site of EGFRvIII-ribozyme is indicated by the arrow.

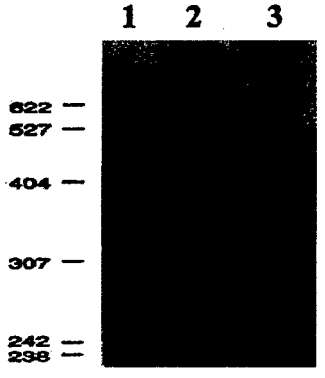

FIGURE 2 – Catalytic activity of EGFRvIII-ribozyme in an extracellular system. *Lane 1*: molecular weight marker. *Lane 2*: ^{32}P-labeled EGFRvIII transcripts with expected size of 715 nucleotides. *Lane 3*: cleavage products of the EGFRvIII-ribozyme (455 and 260 nucleotides).

Intracellular model system for studying the specificity and efficacy of EGFRvIII ribozyme

We next investigated the catalytic activity of these ribozymes intracellularly. The complexity of heterodimerization and transphosphorylation between the EGF family receptors in breast cancer cells makes it difficult to determine the specificity of this EGFRvIII ribozyme. Furthermore, the action of this ribozyme is to interrupt EGFRvIII gene expression. One of our early studies have demonstrated that the expression of EGFRvIII in MCF-7 human breast cancer cells induced cell proliferation and enhanced tumorigenicity in nude mice.[12] We selected 2 cell lines, MCF-7/EGFRvIII and MCF-7/LCC2, as our model systems. MCF-7/EGFRvIII was established by stable transfection of EGFRvIII in MCF-7 cells and appears to express high levels of EGFRvIII and very low levels of wild-type EGFR. Expression of EGFRvIII in MCF-7 cells induces cell proliferation and enhanced tumorigenicity in nude mice.[12] MCF7/LCC2 is a stepwise *in vitro* selection of the hormone-independent human breast cancer variant MCF-7 against 4-hydroxytamoxifen. MCF7/LCC2 cells appear to express moderate to high levels of wild-type EGFR, but do not express detectable levels of EGFRvIII.[16] The expression of ErbB-2, ErbB-3 and ErbB-4 levels in MCF-7/EGFRvIII and MCF7/LCC2 are comparable to the untransfected MCF-7 cells. We therefore used MCF-7/EGFRvIII to examine the intracellular efficacy and efficiency of this EGFRvIII ribozyme and MCF-7/LCC2 was used as a control to test the specificity of this EGFRvIII ribozyme.

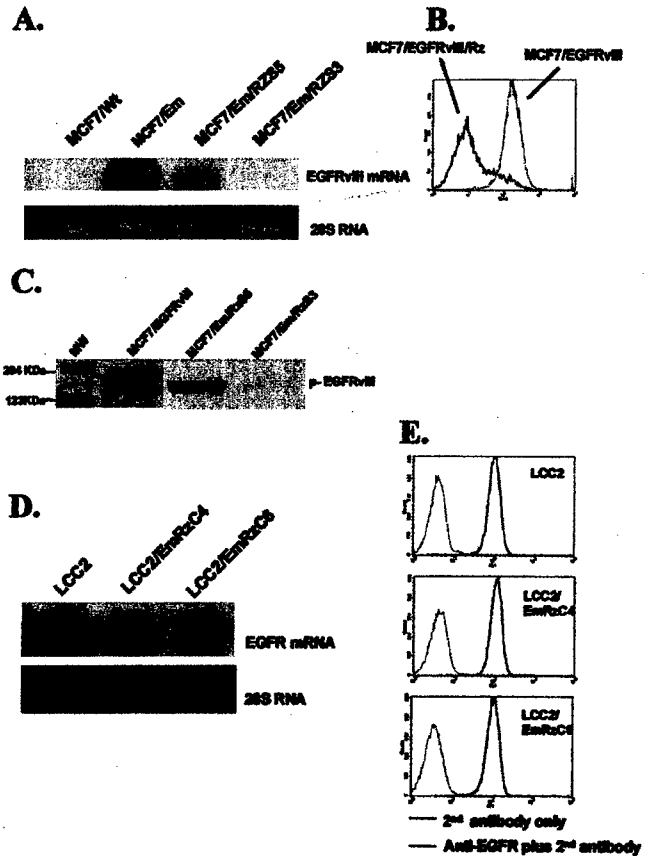

FIGURE 3 – Ribozyme mediated downregulation of EGFRvIII in both mRNA and protein level in MCF7/EGFRvIII/Rz cells. EGFRvIII ribozyme has neither affect on wild-type EGFR mRNA nor EGFR protein expression in EGFRvIII ribozyme transfected MCF7/LCC2 breast cancer cells. *a*: Northern analysis illustrated ribozyme significantly reduced EGFRvIII mRNA expression levels in MCF/Em/RzS5 cells and completely suppressed the EGFRvIII mRNA expression in MCF/Em/RzS3 cells. 28S RNA was used as loading control. *b*: Ribozyme mediated downregulation of EGFRvIII protein expression levels in MCF-7/EGFRvIII/Rz cells was quantitatively measured by flow cytometry. *c*: EGFRvIII autophosphorylation was correlated with reduced protein levels in EGFRvIII-Rz transfected cells. Two micrograms of cell lysates were immunoprecipitated with a specific anti-EGFRvIII antibody. Precipitated proteins were then subjected to Western blotting with an anti-phosphotyrosine antibody. *d*: Northern analysis of EGFR mRNA expression, 28S RNA was used as loading control. *e*: FACS analysis of EGFR expression with anti-EGFR antibody.

We constructed this EGFRvIII ribozyme in a mammalian expression vector pCDNA3.1/Zeo under a CMV promoter control. We then transfected the EGFRvIII ribozyme (Rz) into MCF-7/EGFRvIII and MCF-7/LCC2 cells. Stable transfectants were selected and denoted as MCF-7/Em/Rz and LCC2/Rz. Northern analysis was carried out to evaluate the enzyme activity of this EGFRvIII ribozyme. As shown in Figure 3*a*, this novel EGFRvIII ribozyme was capable of downregulating EGFRvIII mRNA expression significantly in clone MCF-7/Em/RzS5, and completely eliminating the EGFRvIII mRNA expression in clone MCF-7/Em/RzS3. In contrast, no effects were observed on wild-type EGFR mRNA expression in EGFRvIII Rz transfected LCC2 cells respectively (Fig. 3*d*). These results clearly demonstrate EGFRvIII ri-

bozyme to be capable of downregulating EGFRvIII mRNA, and have no effect on wild-type EGFR mRNA intracellularly.

To further characterize the ribozyme effect, we quantitatively examined the EGFRvIII ribozyme mediated downregulation of EGFRvIII receptor protein expression in these EGFRvIII-ribozyme transfected cells by FACS analysis. Figure 3B depicts MCF-7/EGFRvIII cells expressing high levels of EGFRvIII receptor. EGFRvIII-ribozyme almost completely downregulates EGFRvIII expression in ribozyme transfected MCF-7/EGFRvIII cells (Fig. 3*b*). In addition, the level of EGFRvIII phosphorylation is reduced correlating with downregulation of EGFRvIII protein levels in MCF-7/EGFRvIII cells (Fig. 3*c*). In contrast, no effect on the wild-type EGFR expression that was observed in EGFRvIII ribozyme transfected MCF-7/LCC2 cells (Fig. 3*e*). These experiments demonstrate the efficacy, efficiency and specificity of this EGFRvIII ribozyme intracellularly. These data suggest that the constructed EGFRvIII Rz is biologically functional ribozyme.

Ribozyme-mediated downregulation of endogenous EGFRvIII in human breast cancer cells

To investigate the biological activity of this EGFRvIII ribozyme on endogenous EGFRvIII and the biological significance of EGFRvIII in human breast cancer, we selected the human breast cancer cell line, MDA435/LCC6. MDA435/LCC6 cell line was selected for growth as an ascites tumor in athymic mice from untransfected MDA-MB-435 human breast cancer cell line.[17] MDA435/LCC6 cells grow as both malignant ascites and solid tumours *in vivo* in nude mice and nude rats, with a tumor incidence of approximately 100%.[17] MDA435/LCC6 cells also retain the anchorage-dependent and anchorage-independent *in vitro* growth properties of the un-transfected MDA-MB-435 cell.[17] MDA435/LCC6 cell line expresses low levels of endogenous EGFRvIII, but it does not express detectable levels of wild-type EGFR. EGFRvIII-ribozyme, as well as an empty vector (mock transfected), was introduced into this cell line by stable transfection. The sublines were established and designated as LCC6/Rz. We then assessed the ribozyme-mediated downregulation of EGFRvIII expression as well as other EGF-family receptors by FACS analysis. Figure 4*a* illustrates that EGFRvIII ribozyme has no effect on the expression of ErbB-2 and ErbB-3, whereas the EGFRvIII expression was completely abolished. As shown in Figure 4*b*, EGFRvIII mRNA is almost un-detectable in LCC6/RzC1 and LCC6/RzC9 transfectants compared to LCC6 un-transfected cells. No significant effect was observed in clone LCC6/RzC7.

Downregulation of EGFRvIII in cell lines expressing endogenous egfrviii results in an inhibition of growth rate and colony formation

The biological effect of downregulation of EGFRvIII expression by EGFRvIII-ribozyme in MDA435/LCC6 cells was evaluated by anchorage-dependent and anchorage-independent growth assays. Downregulation of EGFRvIII expression in the MDA435/LCC6 human breast cancer cell line resulted in a significant inhibition of growth rate compared to their un-transfected cell ($p < 0.0001$) (Fig. 5*a*). Inhibition of colony formation was independent of colony size ($p < 0.0001$) (Fig. 5*b*). In comparison with ribozyme transfected MCF-7/LCC2 cells, no difference in growth rate on ribozyme transfected MCF-7/LCC2 cells (MCF-7/LCC2/Rz) *vs.* un-transfected MCF-7/LCC2 cells were observed as expected (data not shown). These results indicate a partial reversion of transformation by downregulation of EGFRvIII in MDA435/LCC6 cells.

Antitumor activity of EGFRvIII-ribozyme in breast cancer cells

We next explored the *in vivo* effects of downregulation of EGFRvIII expression in MDA435/LCC6 cells. Un-transfected MDA435/LCC6 cells (1×10^6 cells), as well as the mock transfected or ribozyme-transfected cells, were implanted in nude mice. The MDA435/LCC6 un-transfected and mock transfected cells grew to a mean tumor size of 435.4 ± 39 mm.^{3}. In contrast, tumor

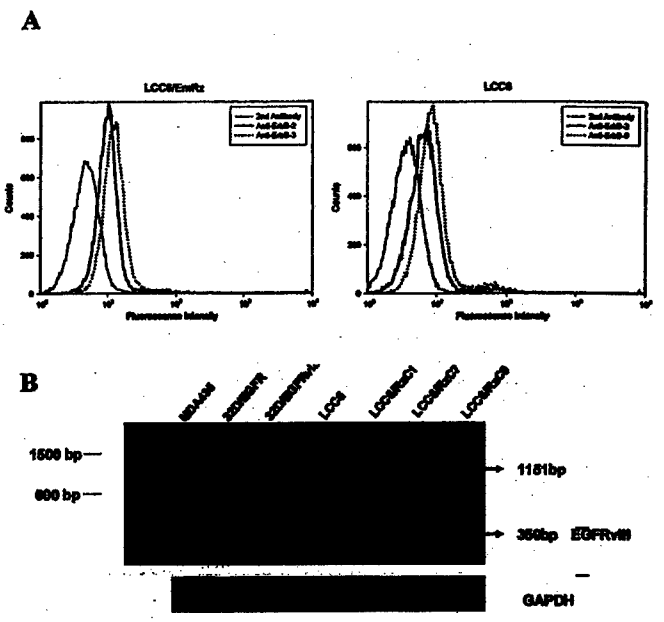

FIGURE 4 – *a*: EGFRvIII ribozyme has no effect on the expression of ErbB-2 and ErbB-3. The level of ErbB-2 and ErbB-3 in MDA435/LCC6 un-transfected cells and the ribozyme-transfected MDA435/LCC6 clones were quantitatively measured by flow cytometry. *b*: Downregulation of endogenous EGFRvIIImRNA by EGFRvIII ribozyme. The expression of EGFRvIII mRNA was detected by RT-PCR. RNA isolated from EGFRvIII and EGFR transfected 32D cells were used as positive control RNAs isolated from MDA-MB-435 cells was used as negative control. As illustrated in this ethidium bromide gel, the EGFR mRNA (1,151 bp) and EGFRvIII mRNA (350 bp) as detected in EGFR and EGFRvIII transfected 32D cells, respectively.

growth of ribozyme-expressing MDA435/LCC6 cells was significantly inhibited with $p < 0.0001$ (Table I). To further confirm the inhibition of tumorigenicity is due to the downregulation of EGFRvIII expression in LCC6/Rz cells, we evaluated the EGFRvIII expression of the xenograft tumors. Lysates obtained from homogenized xenograft tumors of the untransfected MDA435/LCC6 and ribozyme transfected MDA435/LCC6 cells were subjected to Western blot analysis of EGFRvIII expression with a specific EGFRvIII monoclonal antibody. As shown in Figure 6, EGFRvIII expression was substantially reduced in LCC6/Rz transfectants comparing with the un-transfected MDA435/LCC6 cell. These results clearly demonstrate that EGFRvIII ribozyme is biological active in athymic nude mice, and effectively repressed EGFRvIII expression in breast cancer xenografts.

DISCUSSION

Epidermal growth factor receptor mutant III (EGFRvIII) is a rearrangement, ligand-independent, constitutively active EGFR variant, and a tumor-associated receptor. EGFRvIII involves an in-frame deletion between nucleotides 275–1075 in the normal EGFR gene sequence, which corresponds to a deletion of 267 amino acids in the EGFR extracellular domain and distinguishes it from full-length EGFR.[1] It has been detected in brain, lung, ovarian, breast and prostate cancer,[2–7] and has not been observed from normal adult tissues.[8,9] In our early studies, we demonstrated that high incidence of human primary invasive breast cancer tissues express both EGFRwt and EGFRvIII mRNA in the same tumor, but there is no detectable level of EGFRvIII mRNA in normal breast tissue.[11] These unique features of EGFRvIII make it an excellent target for biologically based therapies. Interestingly, as a result of the deletion of 801 base pairs, the fusion junction creates a novel amino acid glycine, which is transcribed subse-

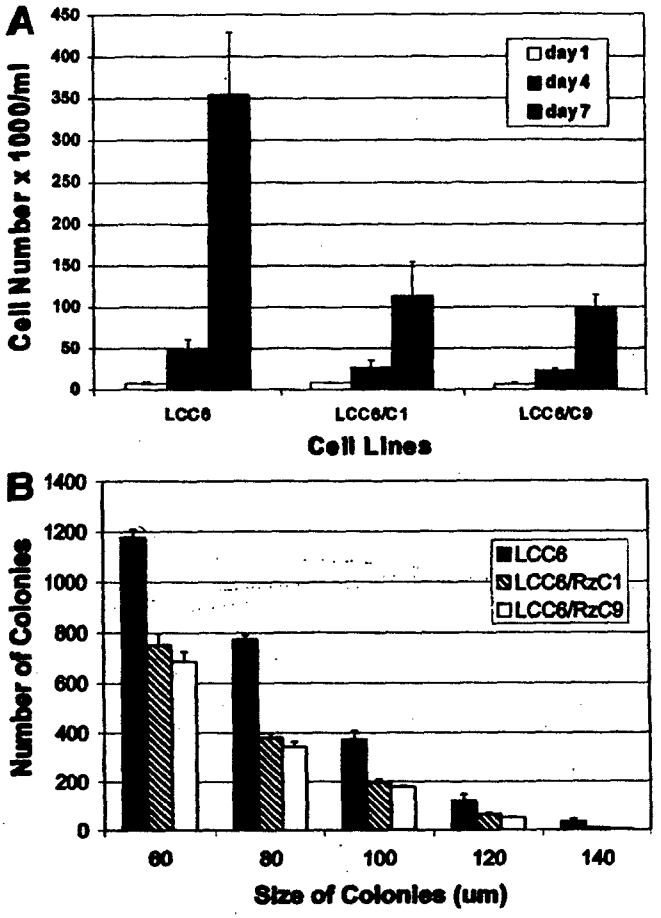

FIGURE 5 – Cell growth assays. *a*: Anchorage-dependent growth assay. The expression of EGFRvIII ribozyme in MDA435/LCC6 human breast cancer cells resulted in an inhibition of 40% growth rate compared to their un-transfected cell. Cells were plated at a density of 3×10^3 cells/ml. Viable cells were counted on Day 1, Day 4 and Day 7 after seeding. All samples were prepared in triplicate and this assay was repeated three times. *b*: Anchorage-independent growth assay showed that the inhibition of colony formation was independent of colony size. A bottom layer of 1 ml IMEM containing 0.6% agar and 10%FBS was prepared in 35-mm tissue culture dishes. After the bottom layer solidified, cells (10,000 cells/dish) were then added in a 0.8 ml top layer, containing 0.4% Bacto Agar and 5% FBS. The cells were incubated for about 10 days at 37°C. Colonies larger than 60, 80,100, 120 and 140 um were counted by a cell colony counter. All samples were prepared in triplicate. The assay was repeated 2 times.

TABLE I – ANTI-TUMOR-ACTIVITY OF EGFRvIII-RIBOZYME IN LCC6 BREAST CANCER CELLS AT DAY 25 POST-INOCULATION[1]

Cell line	Size of tumor (mm^3)
LCC6/vector	435.39 ± 39.0
LCC6/EmRzC1	216.96 ± 79.1
LCC6/EmRzC9	158.37 ± 50.07[2]

[1] 1×10^6 cells/site were injected in 4–6 week old female athymic nude mice–[2] $p < 0.0001$.

quently into a ribozyme target codon GUA. We therefore generated a hammerhead ribozyme targeting this EGFRvIII novel fusion junction. Although the flanking sequences of this ribozyme can bind to both EGFRwt and EGFRvIII mRNAs, the EGFRwt mRNA does not obtain this GUA ribozyme cleavage site. Thus, this ribozyme is only able to cleave the EGFRvIII mRNA but not the wild-type EGFR mRNA. We demonstrated that this EGFRvIII

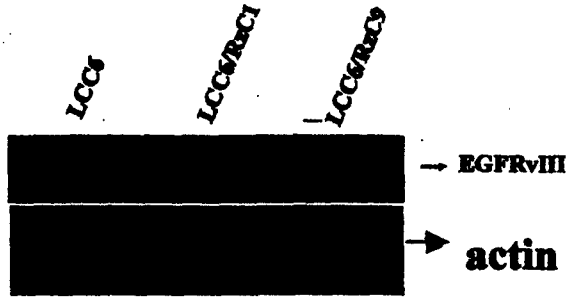

FIGURE 6 – Detection of ribozyme downregulation of EGFRvIII expression *in vivo*. Lysates prepared from tumors derived from MDA435/LCC6 and LCC6/Rz xenografts were subjected immunoblotted analysis for EGFRvIII expression with an anti-EGFRvIII (Ab-3) antibody. β-Actin expression (46 kDa) was re-probed to indicate evenness of loading protein extract from each sample.

ribozyme effectively catalyzes the precise cleavage of EGFRvIII mRNA, whereas it had no effect on wild-type EGFR and other members of the EGF receptor family under physiological conditions in an extracellular system (Fig. 2).

We further verified the specificity and efficacy of the anti-EGFRvIII-ribozyme in intracellular model systems with MCF-7/EGFRvIII and MCF-7/LCC2 cells. As shown in Figure 3, this EGFRvIII ribozyme substantially downregulates both EGFRvIII mRNA and protein levels in MCF-7/EGFRvIII cells and has no effect on the expression level of wild-type EGFR and other EGF-family receptors. These results clearly demonstrate the high specificity and efficacy of this EGFRvIII Rz. To further assess the efficiency of EGFRvIII-ribozyme and elucidate the biological role of EGFRvIII in breast cancer, we carried out a series of experiments targeting the endogenous EGFRvIII in breast cancer cells. We evaluated the effects of ribozyme-mediated downregulation of EGFRvIII in an EGFRvIII positive human breast cancer cell line, MDA435/LCC6 cells. We observed that the EGFRvIII mRNA and protein was completely abolished. Downregulation of the EGFRvIII in LCC6/Rz cells resulted in a reduction of colony formation in an anchorage-independent assay compared to untransfected and mock transfected cells. In addition, downregulation of EGFRvIII in MDA435/LCC6 cells significantly inhibited tumor formation in athymic nude mice (Table I). In comparison, no growth effect was observed in ribozyme transfected MCF-7/LCC2 cells, respectively. This data demonstrated that inhibition of growth is correlated with the level of downregulation of EGFRvIII in these ribozymes transfected cells. Reduction of colony formation and tumorigenicity suggest that EGFRvIII may play a role in MDA435/LCC6 cell proliferation. In addition, this ribozyme was capable of downregulation of endogenous level of EGFRvIII in breast cancer cells.

Despite the high frequency of EGFRvIII expression in primary invasive breast cancer specimens, we were unable to detect the EGFRvIII expression in most of human breast cancer cell lines. Similar observations were reported in human glioblastoma. Virtually, all cell lines derived from primary glioma tumor lose EGFRvIII expression in tissue culture.[18] This suggests either a growth disadvantage *in vitro* or a selection for EGFR overexpression *in vivo*.[19] To further provide the evidence to support this assumption, we evaluated the EGFRvIII expression in MDA435/LCC6 xenografts and MDA435/LCC6 tissue cultured cells. Indeed, we were able to detect substantially higher levels of EGFRvIII expression in the lysate obtained from the MDA435/LCC6 xenografts in comparison with the lysate obtained from the tissue culture of the same breast cancer cell line MDA435/LCC6 by western blotting. EGFRvIII ribozyme significantly downregulate the EGFRvIII expression in ribozyme transfected MDA435/LCC6 xenografts (Fig. 6). These observations further suggest that we have obtained a biological activated and efficient ribozyme. In addition, we also

provide the first evidence that a growth disadvantage for EGFRvIII expression occurs in tissue culture. It implies that for some unknown reason, the EGFRvIII expression is enhanced and triggered in the *in vivo* environment during the tumor progression.

Two other recent studies have examined the effects of an anti-EGFRvIII hairpin ribozyme.[20,21] Both studies demonstrated the ribozymes targeting the exogenous expressed EGFRvIII mRNA. One of the studies was targeting the transfected NIH3T3/EGFRvIII cells and the other study was targeting the EGFRvIII transfected glioblastoma U87 cells. To our knowledge, the studies reported here is the first demonstration of the therapeutic efficacy of an anti-EGFRvIII hammerhead ribozyme targeting the endogenous EGFRvIII expression against human breast cancer cells *in vitro* and *in vivo*. Nevertheless, downregulation of EGFRvIII expression by ribozyme-mediated specific cleavage of EGFRvIII mRNA in breast cancer cells resulted in reduction of tumorigenesis *in vivo* is an elegant approach. The rationale for targeting EGFR for human cancer treatment is now firmly established and numerous clinical trials are in progress. Ribozyme-mediated targeting of EGFRvIII expression as an anticancer strategy appears highly attractive, because EGFRvIII protein is only detected in human breast tumor cells, but not in normal or benign tumors. Therefore, this anti-EGFRvIII ribozyme will only target the breast cancer cells but not the normal cells. Although, its therapeutic use is currently limited by the lack of methodologies to efficiently deliver the ribozyme into the tumor cells, the potential utilization of ribozyme targeting of EGFRvIII may constitute a potential future promising gene therapeutic approach for a molecular defined subgroup of EGFRvIII expressing breast cancer.

ACKNOWLEDGEMENTS

This work was supported by grants from the United States Army Medical Research and Material Command Breast Cancer Grant DAMD 17-99-1-9206 and The Susan G. Komen Breast Cancer Foundation 9806 to C.K.T. We gratefully acknowledge Ms. E.L. Ealley at the Lombardi Cancer Research Center Animal Shared Resource Facility carried out *in vivo* studies and biostatistician Rebecca Slack at the Lombardi Cancer Research Center Biomathematics and Biostatistics Core carried out the statistical analysis in our study. The FACS analysis data shown in Figures 3*b*, 3*e* and 4*a* were supported in part by the Lombardi Cancer Research Center Flow Cytometry Core Facility. In addition, the ribozyme sequence was verified by the Lombardi Cancer Research Center Macromolecular Analysis Share Resource. Table I xenografts were supported by the Lombardi Cancer Research Center Animal Shared Resource Facility and Figure 5 and Table I were supported by the Lombardi Cancer Research Center Biomathematics and Biostatistics Core Facility, DHHS/NIH Grant P30-CA-51008. We express our great appreciation to Dr. M.E. Lippman for helpful discussions.

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